HPTLC and GC/MS Study of Amaryllidaceae Alkaloids of Two *Narcissus* Species

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In this article, we report on the alkaloid profile and dynamic of alkaloid content and diversity in two *Narcissus* plants at different stages of development. The alkaloid profile of the two *Narcissus* species was investigated by GC/MS and HPTLC. Fifty eight Amaryllidaceae alkaloids were detected, and 25 of them were identified in the different organs of *N. tazetta* and *N. papyraceus*. The alkaloid 3-*O*-methyl-9-*O*-demethylmaritidine is tentatively identified here for the first time from the Amaryllidaceae family, and four alkaloids (tazettamide, sternbergine, 1-*O*-acetyllycorine, 2,11-didehydro-2-dehydroxylycorine) are tentatively identified for the first time in the genus *Narcissus*. The different organs of the two species analyzed showed remarkable differences in their alkaloid pattern, type of biosynthesis, main alkaloid and number of alkaloids. Lycorine-type alkaloids dominated the alkaloid, metabolism in *N. papyraceus*, while alkaloids of narciclasine-, galanthamine- and homolycorine-types were found only in the species *N. tazetta* L.

1. Introduction. – The taxonomy of the genus *Narcissus* is complex and unsettled because of its very varied wild populations, the ease with which hybridization occurs naturally, accompanied by extensive cultivation, breeding, and selection. Hybridization has become very popular, resulting in thousands of commercial *Narcissus* cultivars that are in most cases larger than their wild parents [1]. Thus, selective and efficient analytical methods are required for authentication of the plant material [2]. Meanwhile, knowledge of the complete alkaloid pattern is of interest not only phytochemically, but also in relation to aspects of alkaloid biogenesis and metabolism [3]. Investigation of the alkaloid production and accumulation in plant organs during different stages of development provides important information regarding the optimal time of collecting plant material [4]. *Narcissus* is of interest, since more than 300 alkaloids have been isolated from the genus, and most of them possess some biological activities [5]. There are only few data on the ontogenic variations and distribution of alkaloids in the species of the Amaryllidaceae family, and some results have been obtained in the *Narcissus* species, such as *N. assoanus* and *N. confusus* [6][7].

Several chromatographic methods have been used for studying alkaloid patterns and/or quantities in various biological samples, such as thin-layer chromatography, high performance thin-layer chromatography/densitometry (HPTLC/densitometry), gas chromatography (GC), and gas chromatography/mass spectrometry (GC/MS) [4].

GC/MS is a useful and reliable method for rapid separation and identification of complex mixtures of alkaloids [2][8]. In this respect, using double fingerprinting by combining two chromatographic methods gives a more reliable picture about the studied extracts. It is even more useful when these fingerprints complete each other's missing information, as in the case of HPTLC, which is a reliable quantitative method to assess the quantity of alkaloids in each organ and in different flowering stages, however, in the absence of a standard reference for all the alkaloids present in each extract, it lacks information about the identity of such alkaloids. In addition, the number of samples is limited due to the size of the plate. On the other hand, GC/MS gives a reliable identification of most alkaloids by direct comparison to standards information from databases, with the aid of the retention index, molecular ion peak, and other fragmentation peaks [9].

In the present study, a GC/MS procedure was applied for the first time for the identification and comparison of alkaloidal content of the $CHCl_3$ extracts of roots, aerial parts, and bulbs of *N. tazetta* and the $CHCl_3$ extracts of roots, scales, stem discs, and bulbs of *N. papyraceus* at different flowering stages. To the best of our knowledge, there are no reports on ontogenic variability of the secondary metabolism in these *Narcissus* species. Furthermore, double fingerprinting was achieved by the application of a HPTLC analysis which is considered a valuable tool for reliable identification which provides chromatographic fingerprints that can be visualized and stored as electronic images. HPTLC finger-print analysis has become the most potent tool for quality control of herbal medicines because of its simplicity and reliability [10].

Another aim of this work was to investigate the alkaloid metabolism in different organs of the two *Narcissus* species. We subjected the un-derivatized alkaloid mixture to GC/MS analysis. Most of the alkaloids seem to be suitable for GC, although haemanthamine and homolycorine partly decomposed under the GC conditions used [11][12].

Our study lead to the detection of a probable new alkaloid reported for the first time. Four alkaloids are identified for the first time in the genus *Narcissus*, eight alkaloids are reported for the first time in *Narcissus tazetta* L., and two alkaloids are reported for the first time in *Narcissus papyraceus*.

2. Results and Discussion. – 2.1. *High Performance Thin Layer Chromatography* (HPTLC) *Analysis*. The preliminary HPTLC screening of $CHCl_3$ extracts of bulbs, roots, scales, stem discs, and flowers of *N. papyraceus* (*Fig. 1*) revealed the presence of six different alkaloids with different R_f values ranging from 0.01 to 0.88. In general, higher diversity of alkaloids is observed in bulbs and flowering stem discs, followed by scales and stem discs before flowering, then roots and flowers, and finally stem discs after flowering. Meanwhile, the HPTLC analysis of different $CHCl_3$ extracts of *N. tazetta* organs (*Fig. 2*) showed the presence of nine different alkaloids. The alkaloid with R_f 0.01, as in the case above, is present in all organs.

It is clear from *Figs. 1* and *2* that the alkaloidal profile is different in both *Narcissus* species. However, there are three alkaloids that were found to be common, which occur at R_f values of 0.01, 0.39, and 0.43, the first is common in all the studied extracts, and the one at 0.43 is the reference compound, lycorine.

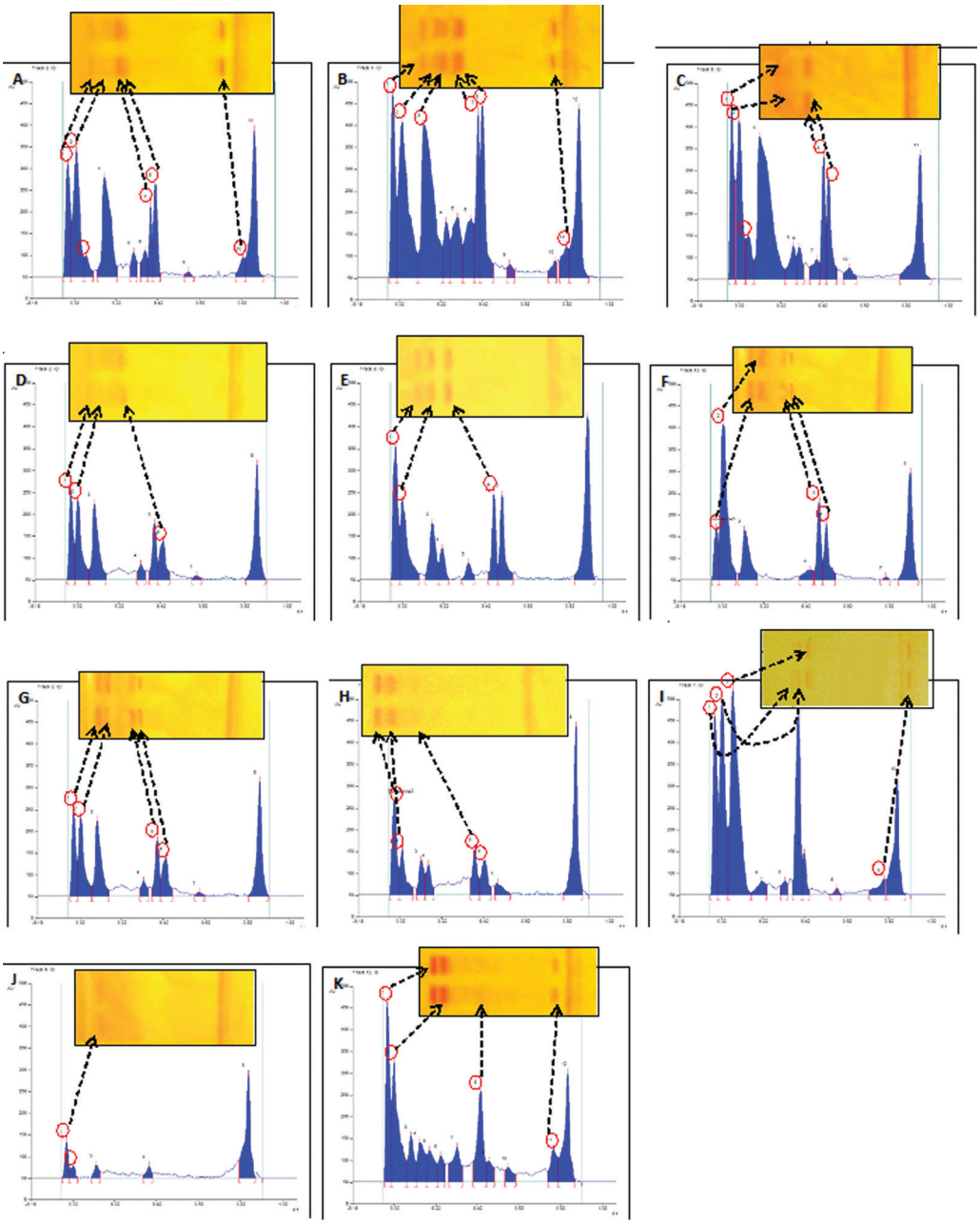

Fig. 1. *HPTLC Scan densitograms at 254 nm and chromatograms after spraying with* Dragendorff*'s spray reagent of the $CHCl_3$ extract of* N. papyraceus. *a*) Bulbs before flowering, *b*) bulbs during flowering, *c*) bulbs after flowering, *d*) roots during flowering, *e*) roots after flowering, *f*) scales before flowering, *g*) scales during flowering, *h*) stem discs before flowering, *i*) stem discs during flowering, *j*) stem discs after flowering, *k*) flowers.

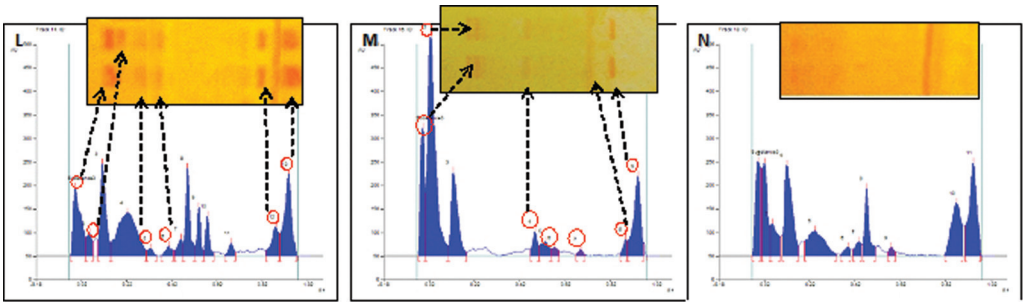

Fig. 2. *HPTLC Scan densitograms at 254 nm and chromatograms after spraying with* Dragendorff*'s spray reagent of $CHCl_3$ extract of* N. tazetta L. *l*) Bulbs, *m*) roots, *n*) aerial parts.

2.2. *GC/MS of the Different Extracts.* Fifty eight compounds found in the different extracts of the two studied *Narcissus* species showed the characteristic mass spectral fragmentation of the Amaryllidaceae alkaloids and their metabolites. Twenty five alkaloids of them (**1**–**25**), belonging to six structural types of Amaryllidaceae alkaloids, in addition to one alkaloid of miscellaneous structure, were identified in the different organs of *N. tazetta* and *N. papyraceus* (*Table 1*). The different organs analyzed showed remarkable differences in respect to their alkaloid pattern, type of biosynthesis, main alkaloid, and number of alkaloids.

To the best of our knowledge, the alkaloid 3-*O*-methyl-9-*O*-demethylmaritidine (**5**) is tentatively identified here for the first time from family Amaryllidaceae. Four alkaloids (tazettamide (**25**), sternbergine (**18**), 1-*O*-acetyllycorine (**19**), 2,11-didehydro-2-dehydroxylycorine (**13**)) were identified for the first time in the genus *Narcissus*. Eight alkaloids (trisphaeridine (**2**), 5,6-dihydrobicolorine (**3**), galanthindole (**9**), anhydrolycorine (**11**), deoxytazettine (**12**), 6-*O*-methylpretazettine (**14**), 11,12-dehydroanhydrolycorine (**15**), and 3-epimacronine(**24**)) are reported for the first time in *Narcissus tazetta* L., and two alkaloids (anhydrolycorine (**11**) and 11,12-dehydroanhydrolycorine (**15**)) are reported for the first time in *Narcissus papyraceus*. Some components remained unidentified due to the lack of reference substances and library spectra.

The EI-MS spectrum of 3-*O*-methyl-9-*O*-demethylmaritidine (**5**; 287 (100, M^+), 272 (42, $[M-Me]^+$), 256 (40, $[M-MeO]^+$), 244 (8), 231 (19), 217 (91), 203 (24), 185 (18), 174 (25), 157 (27), 141 (10), 129 (26), 115 (22), 77 (12), 65 (8)) showed M^+ at *m*/*z* 287, with abundance 100%, a striking feature of the crinine/haemanthamine series alkaloids [13]. It also shows significant peaks at *m*/*z* 272 and 256 due to the expected loss of Me and MeO residues from the parent ion, in addition to other characteristic and diagnostic fragmentation peak. It was observed that most ions have *m*/*z* above 150, thus contain the aromatic ring, which plays an important role in the stabilization of ions [13]. We suggest that the 11,12-bond is broken first, since such a rupture would result in simultaneous release of the strain inherent in the bridgehead system. This leads to the formation of immonium ions that are not in conjugation with the aromatic ring. The resulting ion has the same *m*/*z* value as the molecular ion peak.

The β-5,10b-ethano bridge isomer of alkaloid **5**, 3-*O*-methylmacowine, which shows a similar GC/MS spectrum, is excluded even though the absolute configuration of the

Table 1. *Alkaloids Identified in Different Plant Parts Arranged in Order of Retention Time*

	Alkaloid/Reference(s)	Source[a])	Alkaloid Group	M^+/ base ion (m/z)	t_R	*RI*	First time from Nature	First time from genus	First time from species
1	Ismine [12][17][19]	*N. tazetta* L. Bulbs & roots	Narciclasine	257/238	20.100	2283.2			
2	Trisphaeridine [12][17–19]	*N. tazetta* L. Bulbs, roots & aerial parts	Narciclasine	223/223	20.432	2303.4			+
3	5,6-Dihydrobicolorine [22][23]	*N. tazetta* L. Bulbs & roots	Narciclasine	239/238	21.103	2344.1			+
4	Galanthamine [18][24–26]	*N. tazetta* L. Bulbs & roots	Galanthamine	287/286	22.097	2404.1			
5	3-*O*-Methyl-9-*O*-demethyl-maritidine	*N. papyraceus* Roots (F[b]))	Crinine	287/287	22.980	2458.4	+	+	+
6	*O*-Methylmaritidine [27][28]	*N. tazetta* L. Bulbs & aerial parts *N. Papyraceus* Roots (F), roots (AF[c])), bulbs (BF[d])), bulbs (F), bulbs (AF), Stem discs (BF), stem discs (F), scales (BF), scales (F)	Crinine	301/301	23.133	2467.2			
7	Lycoramine [24]	*N. tazetta* L. Bulbs	Galanthamine	289/288	23.363	2425.5			
8	Vittatine [29]	*N. tazetta* L. Bulbs	Crinine	271/271	23.363	2484.1			
9	Galanthindole [12][30]	*N. tazetta* L. Bulbs & roots	Miscellaneous	281/281	23.728	2503.8			+
10	*O*-Methylpapyramine [27]	*N. papyraceus* Roots (F), bulbs (BF), bulbs (F), bulbs (AF), stem discs (BF), stem discs (F), scales (BF)	Crinine	331/276	23.762	2505.9			
11	Anhydrolycorine [12][20][21][25][26][31]	*N. tazetta* L. Aerial parts, roots & bulbs	Lycorine	251/250	23.855	2511.5			+

Table 1 (cont.)

	Alkaloid/Reference(s)	Source[a])	Alkaloid Group	M^+/ base ion (m/z)	t_R	*RI*	First time from Nature	First time from genus	First time from species
		N. papyraceus Roots (F), Roots (F), Bulbs (BF), Bulbs (F), stem discs (BF), stem discs (F), scales (BF), scales (F)							
12	Deoxytazettine [12][17–19]	*N. tazetta* L. Roots & bulbs	Tazettine	315/231	24.323	2539.7			+
13	2,11-Didehydro-2-dehydroxy-lycorine [20]	*N. papyraceus* Roots (AF) &stem discs (F)	Lycorine	271/250	24.374	2543.2		+	+
14	6-Methylpretazettine [32]	*N. tazetta* L. Roots & bulbs	Tazettine	345/261	25.232	2595			+
15	11,12-Dehydroanhydro-lycorine [31][32]	*N. tazetta* L. Aerial parts, roots, bulbs *N. papyraceus* Roots (F), bulbs (BF), bulbs (AF), stem discs (BF), stem discs (F), scales (BF)	Lycorine	249/24 8	25.427	2607.3			+
16	Papyramine [12]	*N. papyraceus* Roots (F), bulbs (BF), bulbs (AF), stem discs (F), scales (F)	Crinine	317/262	25.792	2629.3			
17	Tazettine [12][19]	*N. tazetta* L. Aerial parts, roots, bulbs	Tazettine	331/247	25.920	2637.2			
18	Sternbergine [24][33]	*N. papyraceus* Bulbs (AF)	Lycorine	331/228	26.803	2690.4		+	+
19	1-*O*-Acetyllycorine [34]	*N. papyraceus* Bulbs (AF)	Lycorine	365/226	26.948	2699.6		+	+

Table 1 (cont.)

	Alkaloid/Reference(s)	Source[a])	Alkaloid Group	M^+/ base ion (m/z)	t_R	*RI*	First time from Nature	First time from genus	First time from species
20	11-Hydroxyvittatine [17][20][25]	*N. tazetta* L. Bulbs	Crinine	287/258	26.956	2700.1			
21	Homolycorine [12][17][21]	*N. tazetta* L. Bulbs and aerial parts	Lycorenine	315/109	27.483	2731.6			
22	Lycorine [12][20][21][26]	*N. tazetta* Aerial parts, roots & bulbs *N. papyraceus* Roots (F), Roots (AF), Bulbs (BF), Bulbs (AF), stem discs (BF), stem discs (F), scales (BF), scales (F)	Lycorine	287/226	27.483	2734.1			
23	8-*O*-Demethyl-homolycorine [12][26]	*N. tazetta* L. Bulbs	Lycorenine	301/109	28.281	2781			
24	3-Epimacronine [20]	*N. tazetta* L. Roots & bulbs	Tazettine	329/245	28.349	2784.7			+
25	Tazettamide [19][35]	*N. tazetta* L. Roots & bulbs	Tazettine	345/260	29.700	2866.8		+	+

[a]) Note: Flowers and Stem disc (AF) were too dilute; repetition done, but just column bleeding observed, both were excluded. [b]) F = flowering stage. [c]) AF = after the flowering stage. [d]) BF = before the flowering stage.

5,10b-ethanobridge cannot be established by GC/MS alone, because the lack of crinine-type alkaloids with β-5,10b-ethano bridge configuration in the genus *Narcissus*, which only has representatives of the α-5,10b-ethano bridge series (hemanthamine type), is a very significant taxonomic feature [1]. Therefore, and by comparing data with assigned library, the structure of **5** was assigned as 3-*O*-methyl-9-*O*-demethylmaritidine.

2.3. *Alkaloid Profile of the Different Organs of* Narcissus papyraceus *at Different Developmental Stages.* Thirty five alkaloids were detected in all the organs of *N. papyraceus* in the different flowering stages, ten of which were identified belonging to two groups of Amaryllidaceae alkaloids namely crinine- and lycorine-types. These involve *ortho-para'* and *para-para'* oxidative coupling of *O*-methylnorbelladine, in their biosynthetic pathway. Lycorine-type alkaloids (especially lycorine (**22**), the most common alkaloid found in Amaryllidaceae plants [15]), dominated the alkaloid metabolism in *N. papyraceus*, with the exception of the extract of bulbs during the flowering stage, which showed a different alkaloid profile in which *O*-methylmaritidine (crinine series) dominated the alkaloid metabolism (*Scheme*). On the other hand, crinine-type alkaloids were also present in relatively high levels in the fractions from *N. papyraceus*, except for roots after the flowering stage which contain minor amounts of crinine-type alkaloids. Bulbs are rich in lycorine-type alkaloids, such as the bisdehydrogenated compound **11** and the bisdehydrogenated derivative thereof, **15**. It is worth mentioning that the bulbs AF also contain lycorine-series alkaloids with unique AcO substituents, compounds **18** and **19**, which were not found in any other organ at any stage of flowering (*Scheme*). Although the alkaloids are at their maximum during the flowering stage, unexpectedly, the flowering stage has the least number of alkaloids, as only ten (three identified) were present during flowering. This finding could convey that alkaloid biosynthesis is at a high rate before and after flowering, however during the flowering stage the biosynthesis slows down in the bulbs, and the alkaloids are translocated in large amounts from other organs (such as roots) which are active during flowering. The absence of alkaloids that require additional biosynthetic steps during the flowering stage further supports our conclusion.

Roots of *N. papyraceus* exhibited a different alkaloid profile in comparison to those of the bulbs, scales, and stem discs. The roots of the growing plant during the flowering stage have di- (**5**), tri- (**6**), and tetra- (**10**, **16**) substituted alkaloids of the crinine series, as well as dehydrated alkaloids of the lycorine series (**11**, **15**). Fourteen alkaloids (seven identified) were detected during flowering, which could be ascribed to the intensive biosynthesis at this stage of development [16] in the roots. In comparison, the roots of senile plants (AF), when the alkaloid biosynthesis is considerably slower [16], showed only five compounds, four of which were identified (**6**, **11**, **15**, and **22**; *Table 2*).

2.4. *Alkaloid Profile of the Different Organs of* Narcissus tazetta *L. Narcissus tazetta* L. extracts showed a greater degree of diversity compared to *N. papyraceus.* Our analysis of the species reported 27 alkaloids, 20 of which were identified belonging to six different types of Amaryllidaceae alkaloids, namely narciclasine-type, galanthamine-type, crinine-type, lycorine-type, tazettine-type, and homolycorine-type, in addition to galanthindole (**9**), which is classified as 'miscellaneous'. Different *N. tazetta* L. organs biosynthesize alkaloids which are all products of oxidative couplings which can occur in *para-ortho*', *para-para*', and *ortho-para* fashion.

Scheme. *Alkaloids Present in the Different Organs of* N. papyraceus *and* N. tazetta *L. Demonstrated on the Biosynthetic Pathways of Amaryllidaceae Alkaloids.* The numbers of the alkaloids are identical with those in *Tables 1* and *2*.

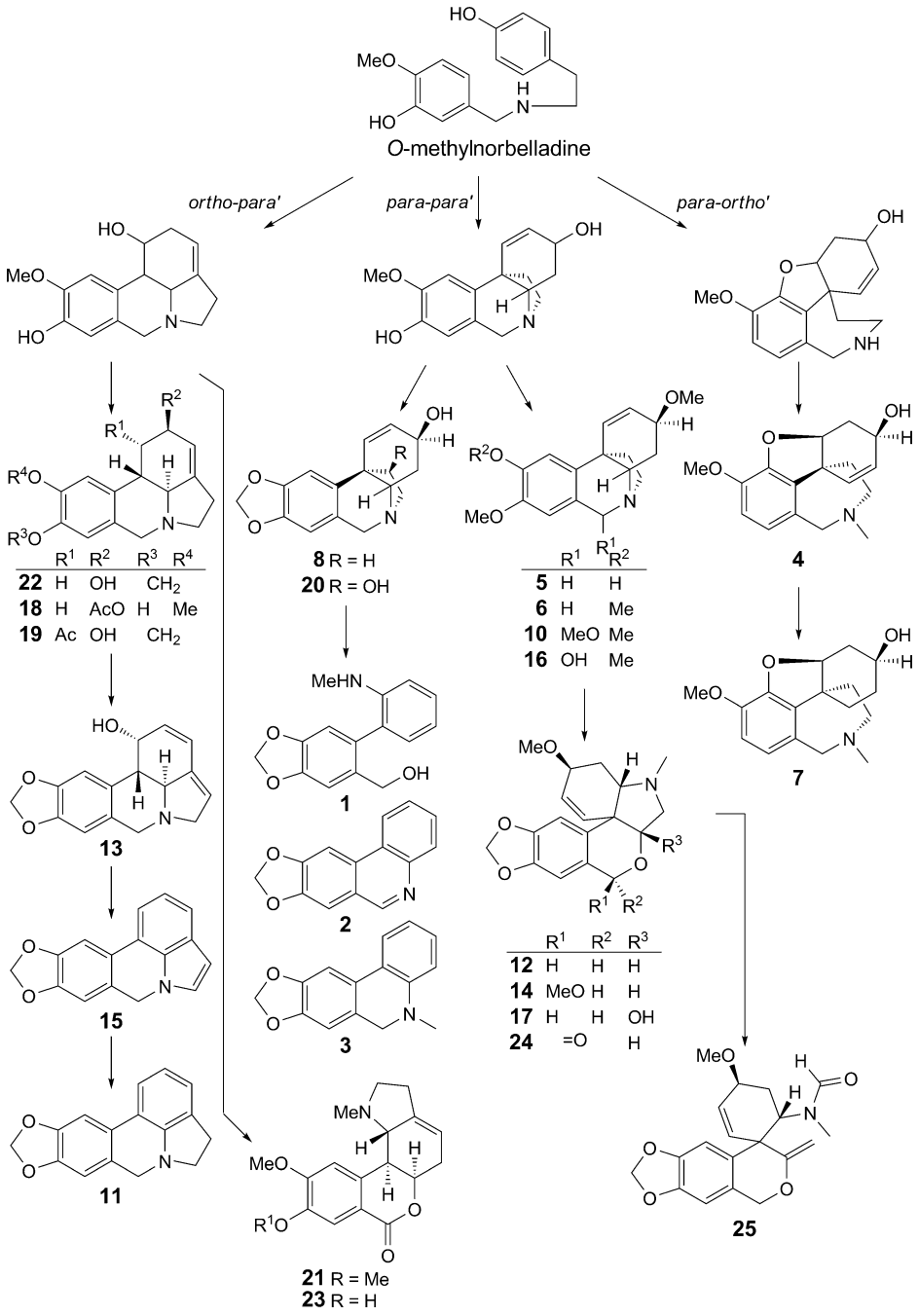

Table 2. *Alkaloid Profile of the Studied Extracts*

Plant extract		Major class	Major alkaloid	Total no. of alkaloids (no. identified)	Biosynthetic pathways	Types of alkaloids	Alkaloid
PW Bulbs[a])	BF[b])	Lycorine	Lycorine (**22**)	16 (6)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
	F[c])	Crinine	*O*-Methylmaritidine (**6**)	10 (3)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O$	**11**
	AF[d])	Lycorine	Lycorine (**22**)	15 (7)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
					ortho-para'	[AcO]	**18**, **19**
PW Stem Disc	BF	Lycorine	Lycorine (**22**)	9 (5)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
	F		Lycorine (**22**)	13 (7)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O-OH$	**13**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
PW Roots	F	Lycorine	Lycorine	14 (7)	*para-para*'	Di [MeO]	**5**
					para-para'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
	AF		Lycorine (**22**)	5 (4)	*para-para*'	Tri [MeO]	**6**

Table 2 (cont.)

Plant extract		Major class	Major alkaloid	Total no. of alkaloids (no. identified)	Biosynthetic pathways	Types of alkaloids	Alkaloid
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2$ H	**15**
PW Scales	BF	Lycorine	Anhydrolycorine (**11**)	13 (6)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					para-para'	Tetra [MeO]	**10**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2$ H	**15**
	F		Lycorine (**22**)	7 (5)	*para-para*'	Tri [MeO]	**6**
					para-para'	Tri [MeO]+[OH]	**16**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2$ H	**15**
N. tazetta L.	Bulb	Tazettine	Tazettine (**17**)	22 (18)	*para-para*'	$-3\ H_2O$ & Ring cleavage	**1**
					para-para'	$-3\ H_2O-$[=O]	**2**, **3**
					para-para'	Tri [MeO]	**6**
					para-para'	+[OH]	**20**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2$ H	**15**
					para-para'	$-H_2O$	**12**
					para-para'	$-H_2O$+Me	**14**
					para-para'	$-H_2O$+[=O]	**24**
					para-para'	$-H_2O$+[=O]+amide	**25**
					ortho-para'	−Me	**23**
	Root	Lycorine	Lycorine (**22**)	18 (13)		Miscellaneous	**9**
					para-para'	$-3\ H_2O$ & Ring cleavage	**1**
					para-para'	$-3\ H_2O-$[=O]	**2**, **3**
					ortho-para'	$-2\ H_2O$	**11**
					ortho-para'	$-2\ H_2O-2$ H	**15**
					para-para'	$-H_2O$	**12**
					para-para'	$-H_2O$+Me	**14**
					para-para'	$-H_2O$+[=O]	**24**

Table 2 (cont.)

Plant extract		Major class	Major alkaloid	Total no. of alkaloids (no. identified)	Biosynthetic pathways	Types of alkaloids	Alkaloid
					para-para'	$-H_2O+[=O]+$ amide	**25**
						Miscellaneous	**9**
	Aerial	Lycorenine	Homolycorine (**21**)	8 (8)	*para-para*'	$-3\ H_2O-[=O]$	**2**
					ortho-para'	$-2\ H_2O-2\ H$	**15**
					para-para'	Tri [MeO]	**6**

[a]) PW = Paperwhite (*Narcissus papyraceus*). [b]) BF = before the flowering stage. [c]) F = flowering stage. [d]) AF = after the flowering stage.

Alkaloids of narciclasine-, galanthamine-, tazettine-, and homolycorine-types were found only in the studied species *N. tazetta* (*Table 1*, *Scheme*), a finding which could open the field to explore characteristic phytomarkers for this species [11]. *N. tazetta* extracts were found to contain many alkaloids that require multiple steps for their biosynthesis such as ismine (**1**) which is derived from vittatine (**8**). Alkaloid **14**, the 6-methyl derivative of pretazettine, is derived from vittatine or crinine alkaloids, in addition to the alkaloid homolycorine (**21**), which is the oxidation product of lycorenine [1]. Furthermore, *N. tazetta* extracts contain many alkaloids with different types of substitutions (*Table 2*). It is noticeable that tetra-substituted alkaloids belonging to the crinine series (**10**, **16**), which were present in most *N. papyraceus* extracts, are completely absent from all *N. tazetta* L. organs. The alkaloids vittatine (**8**) and 11-hydroxyvittatine (**20**) of the crinine series are unique to the *N. tazetta* L. bulbs, and were absent in all the other studied extracts (*Tables 1* and *2*). *N. tazetta* L. roots showed 18 different alkaloids, 13 of which were identified, distributed in the different alkaloid groups mentioned above, except for crinine and homolycorine type alkaloids. *N. tazetta* L. aerial parts showed five different alkaloids which were identified, each belonging to a different alkaloid group, namely narciclasine-, crinine-, lycorine-, tazettine-, and homolycorine-types. Unexpectedly, the *N. tazetta* L. aerial extract lacks lycorine (**22**).

3. Conclusions. – It is well established that alkaloid profiles of the two studied species vary with time, location, and developmental stage. In many instances, the site of biosynthesis is restricted to a single organ, but accumulation of the corresponding products can be detected in several other plant tissues. Long-distance transport must take place in these instances.

The results of the HPTLC screening revealed that generally bulbs precede the other organs in respect to alkaloid biosynthesis followed by flowers; scales and roots and finally stem discs with the exception to flowering stem discs that have the second highest total content. Therefore, it is probably better to pick up the target organ – when searching for the maximum amount of alkaloids – during the flowering stage. As shown above in the case of bulbs, stem discs, and roots, the same organ had a higher total alkaloid content during flowering than before or after flowering. In contrast to *N. papyraceus*, *N. tazetta* L. roots show the maximum alkaloidal content. Aerial parts are least concerning the total alkaloid content. In conclusion, the roots and bulbs of *N. tazetta* are the main target organs when searching for alkaloids. The HPTLC profile was shown as a useful phytochemical marker of genetic variability in the studied plant population. HPTLC profile differentiation is an important and powerful procedure which has been often employed in placement of the plant in taxonomic categories [17].

Taking into account the complexity of the alkaloid fractions, GC/MS is the method of choice for a rapid analysis of Amaryllidaceae alkaloids. It requires a minimum of plant material and allows the identification of numerous compounds, some of them of pharmacological interest [18][19]. In this respect, populations with alkaloid metabolism containing galanthamine or narciclasine derivatives, such as *N. tazetta* L. organs, and those dominated by lycorine derivatives such as *N. papyraceus* organs, especially its stem discs, could be of interest for further investigations. In addition, many of the other alkaloids found in the studied populations are of pharmacological interest.

The ontogenic stage at the time of collection of plant material is of importance. Significant differences in the alkaloid composition, type of biosynthesis, and number of alkaloids were observed before flowering, at flowering, and after that stage. It is evident that there are substantial changes in the methylation and dehydration of alkaloids at different ontogenetic stages (*Table 2*). We suggest that the biosynthesis of Amaryllidaceae alkaloids in *N. papyraceus* before the flowering stage starts in bulbs and scales, reaches its maximum during the flowering period, mainly in the roots and stem discs, and declines towards the AF phase, where only the bulbs are biosynthetically active. These were found to contain alkaloids with unique substituents during this stage.

Experimental Part

1. *Plant Material.* 1.1. Narcissus papyraceus. Approximately 200 bulbs (about 5.5 kg) of *Narcissus papyraceus* were imported from the Netherlands before the flowering stage. Bulbs were planted in the King Mariout area, Alexandria, Egypt. Bulbs were collected in September (during the preflowering stage; 2.35 kg), immediately chopped, and exhaustively extracted with EtOH. In December (during the flowering stage), half of the planted bulbs were collected (2 kg), chopped, and exhaustively extracted with EtOH. The remaining planted bulbs were finally collected in March, three months after the flowering stage (4.2 kg), and were treated in the same way. Harvesting was carried out in the early morning in all cases. The extracts were concentrated under reduced pressure, acidified with 5% tartaric acid to pH 2, and then defatted with PE, filtered, and then washed with Et_2O. The acidic aq. phase was rendered alkaline with aq. NH_4OH soln. to pH 10, and extracted successively with $CHCl_3$, AcOEt, and BuOH. Stem discs, scales, and roots were treated using the same method during the different flowering stages. TLC Screening of the different solvent extracts showed that most of the alkaloidal content was present in the $CHCl_3$ extracts. Therefore, the $CHCl_3$ extracts of the different organs were used in this study.

1.2. Narcissus tazetta *L.* The plant bulbs (546 g), roots (214 g), and aerial parts (342 g) were collected during the flowering season (January). The three organs were immediately chopped and exhaustively extracted with EtOH. The extracts were concentrated under reduced pressure, acidified with 5% aq. tartaric acid soln. to pH 2, and then defatted with PE, filtered, and then washed with Et_2O. The acidic aq. phase was rendered alkaline with aq. NH_4OH soln. to pH 10, and was then extracted successively with $CHCl_3$, AcOEt, and BuOH. TLC Screening of the different solvent extracts showed that most of the alkaloidal content was present in the $CHCl_3$ extracts. Therefore, the $CHCl_3$ extracts of the different organs were used in this study. The $CHCl_3$ extracts of the studied organs were evaporated to dryness under reduced pressure. Ten mg of each sample was dissolved in 10 ml MeOH; 6 mg of lycorine (**22**) were dissolved in 10 ml of MeOH, and used as a reference.

The two above mentioned plants were kindly identified by Professor *Alam El-Din Noah* (Professor of Floriculture, Ornamental horticulture and landscape design, Faculty of Agriculture, Alexandria University, Egypt). A voucher sample of each was deposited with the Department of Pharmacognosy, Faculty of Pharmacy, Alexandria, Egypt.

2. *Parameters and Conditions.* 2.1. *Gas Chromatography/Mass Spectrometry* (GC/MS). EI-MS were obtained on a GC/MS *Agilent 6890 + MSD 5975* operating in EI mode at 70 eV. A *Sapiens-X5 MS* column (equivalent to *HP-5 MS*; 30 m × 0.25 mm × 0.25 μm) was used. The temp. program was: 100–180° at 15° min^{-1}, 1 min hold at 180°, 180–300° at 5° min^{-1} and 1 min hold at 300°. The injector temp. was 280°. The flow rate of He carrier gas was 0.8 ml min^{-1}. In most cases, the split ratio was 1:20, but with more diluted samples, a split ratio of 1:5 was applied. A hydrocarbon mixture (C9–C36, *Restek*, Cat no. 31614) was used for performing the *RI* calibration. GC/MS Results were analyzed using AMDIS 2.64 software (NIST). The proportion of each compound in the alkaloid fractions was expressed as a percentage of the total ion flow.

The alkaloid identification was performed by comparisons of *RI* (Retention Index) and mass spectra with the information of reference samples. When such samples were not available, tentative structures

were proposed on the basis of the mass spectral fragmentation. The reference compounds correspond to our internal database of Amaryllidaceae alkaloids, which have been isolated from numerous Amaryllidaceae plant species. The *RI* is calculated using a standard alkane mixture, as indicated in the *Material and Methods* section. Although the provided data do not express a real quantification, it can be used for comparison between abundance of the alkaloids [12].

2.2. *HPTLC Analysis.* 2.2.1. *Mobile Phase Composition.* Different compositions of the mobile phase for HPTLC analysis were tested in order to obtain high-resolution and reproducible peaks. The desired aim was achieved using the solvent system $CHCl_3$/MeOH 9 : 1 (double run) with one drop of aq. NH_4OH soln. to avoid tailing. All solvents used were obtained from *Merck*, HPLC grade.

2.2.2. *Sample Preparation and Application.* Ten mg of each sample was dissolved in 10 ml MeOH; 6 mg of lycorine alkaloid were dissolved in 10 ml of MeOH and used as a reference in HPTLC analysis. Sample solns. were applied by means of a *Camag* (Wilmington, NC) *Linomat IV* automated spray-on band applicator equipped with a 100-µl syringe and operated with the following settings: band length 6 mm, application rate 15 s/µl, distance between bands 4 mm, distance from the plate side edge 1 cm, and distance from the bottom of the plate 1 cm. Each sample was applied twice in two consecutive tracks. Samples were applied on pre-coated silica gel *60GF254* aluminum sheets (6.3×20 cm) with the help of *Linomat 5* applicator attached to *Camag* HPTLC system, which was programmed through WIN CATS software.

2.2.3. *Development of Chromatogram.* After the application of spots, the chromatogram was developed in a glass chamber (20×20 cm) saturated with $CHCl_3$ and MeOH in the ratio 9 : 1 for 15 min. After complete drying of the plate, a second run was carried out in the same system for another 15 min. The optimized chamber saturation time for mobile phase was 30 min at r.t. (25°). The developed plate was dried by hot air.

2.2.4. *Detection of Spots.* The air-dried plates were viewed in ultra-violet radiation to mid-day light. Zones were quantified by linear scanning at 254 nm with a *Camag TLC Scanner 3* with a deuterium source in the reflection mode, slit dimension settings of length 6 mm and width 0.4 mm, monochromator bandwidth 20 nm, a scanning rate of 20 mm/sec. The R_f values and fingerprint data were recorded by WINCATS software.

REFERENCES

[1] J. Bastida, R. Lavilla, F. Viladomat, 'Chemical and Biological Aspects of Narcissus Alkaloids', in 'The Alkaloids: Chemistry and Biology', G. A. Cordell, Elsevier, Amsterdam, Vol. 61, 2006, pp. 87–179.
[2] P. Drašar, J. Moravcova, *J. Chromatogr. B* **2004**, *812*, 3.
[3] A. El Bazaoui, M. A. Bellimam, A. Soulaymani, *Fitoterapia* **2011**, *82*, 193.
[4] S. Jakabová, L. Vincze, Á. Farkas, F. Kilár, B. Boros, A. Felinger, *J. Chromatogr. A* **2012**, *1232*, 295.
[5] A. Lubbe, H. Gude, R. Verpoorte, Y. H. Choi, *Phytochemistry* **2013**, *88*, 43.
[6] S. López, J. Bastida, F. Viladomat, C. Codina, *Planta Med.* **2003**, *69*, 1166.
[7] F. Viladomat, J. M. Llabrés, J. Bastida, R. M. Cusidó, C. Codina, *Physiol. Plant.* **1986**, *68*, 657.
[8] M. Kreh, R. Matusch, L. Witte, *Phytochemistry* **1995**, *38*, 773.
[9] S. Berkov, B. Sidjimova, L. Evstatieva, S. Popov, *Phytochemistry* **2004**, *65*, 579.
[10] M. Ram, M. Z. Abdin, M. A. Khan, P. Jha, 'High-Performance Thin-Layer Chromatography (HPTLC)', Springer-Verlag, Berlin, Heidelberg, 2011, p. 105.
[11] R. Gotti, J. Fiori, M. Bartolini, V. Cavrini, *J. Pharm. Biomed. Anal.* **2006**, *42*, 17.
[12] J. P. de Andrade, N. B. Pigni, L. Torras-Claveria, S. Berkov, C. Codina, F. Viladomat, J. Bastida, *J. Pharm. Biomed. Anal.* **2012**, *70*, 13.
[13] P. Longevialle, D. H. Smith, A. L. Burlingame, H. M. Fales, R. J. Highet, *Org. Mass Spectrom.* **1973**, *7*, 401.
[14] A. M. Duffield, R. T. Aplin, H. Budzikiewicz, C. Djerassi, C. F. Murphy, W. C. Wildman, *J. Am. Chem. Soc.* **1965**, *87*, 4902.
[15] N. Unver Somer, D. Cicek Polat, A. Emir, M. A. Onur, G. I. Kaya, *Acta Chromatogr.* **2013**, *25*, 331.

[16] S. Berkov, T. Doncheva, S. Philipov, K. Alexandrov, *Biochem. Syst. Ecol.* **2005**, *33*, 1017.
[17] S. Berkov, J. Bastida, B. Sidjimova, F. Viladomat, C. Codina, *Biochem. Syst. Ecol.* **2008**, *36*, 638.
[18] S. Berkov, L. Evstatieva, S. Popov, *Z. Naturforsch. C* **2004**, *59*, 65.
[19] S. Xiao, S. Qian, Y. Wang, Y. Zhang, Y. Cheng, *Analyst* **2013**, *138*, 1772.
[20] S. Berkov, J. Bastida, R. Tsvetkova, F. Viladomat, C. Codina, *Z. Naturforsch. C* **2009**, *64*, 311.
[21] M. H. Hetta, A. A. Shafei, *J. Am. Sci.* **2013**, *7*, 9.
[22] F. Viladomat, J. Bastida, G. Tribo, C. Codina, M. Rubiralta, *Phytochemistry* **1990**, *29*, 1307.
[23] Y. B. Ji, B. F. Wang, N. Chen, D. X. Song, J. Zheng, Y. Dong, *Appl. Mech. Mater.* **2013**, *414*, 3269.
[24] S. Berkov, F. Viladomat, C. Codina, S. Suárez, A. Ravelo, J. Bastida, *J. Mass Spectrom.* **2012**, *47*, 1065.
[25] S. Berkov, S. Romani, M. Herrera, F. Viladomat, C. Codina, G. Momekov, I. Ionkova, J. Bastida, *Phytother. Res.* **2011**, *25*, 1686.
[26] V. Georgiev, I. Ivanov, S. Berkov, M. Ilieva, M. Georgiev, T. Gocheva, A. Pavlov, *Eng. Life Sci.* **2012**, *12*, 534.
[27] R. Suau, R. Rico, A. I. García, A. I. Gómez, *Heterocycles* **1990**, *31*, 517.
[28] E. E. ElGorashi, J. van Staden, *South Afr. J. Bot.* **2003**, *69*, 593.
[29] F. Conforti, M. R. Loizzo, M. Marrelli, F. Menichini, G. A. Statti, D. Uzunov, F. Menichini, *Pharm. Biol.* **2010**, *48*, 2.
[30] B. B. Sarikaya, S. Berkov, J. Bastida, G. I. Kaya, M. A. Onur, N. U. Somer, *Nat. Prod. Commun.* **2013**, *8*, 327.
[31] I. Ivanov, V. Georgiev, S. Berkov, A. Pavlov, *J. Plant Physiol.* **2012**, *169*, 206.
[32] F. Cabezas, A. Ramírez, F. Viladomat, C. Codina, J. Bastida, *Chem. Pharm. Bull.* **2003**, *51*, 315.
[33] A. Evidente, I. Iasiello, G. Randazzo, *J. Nat. Prod.* **1984**, *47*, 1003.
[34] J. Niño, G. M. Hincapié, Y. M. Correa, O. M. Mosquera, *Z. Naturforsch. C* **2007**, *62*, 223.
[35] J. E. Ortiz, S. Berkov, N. B. Pigni, C. Theoduloz, G. Roitman, A. Tapia, J. Bastida, G. E. Feresin, *Molecules* **2012**, *17*, 13473.

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